Further improvements on the phosphotriester synthesis of deoxyribooligonucleotides and the oligonucleotide directed site-specific mutagenesis of *E. coli* lipoprotein gene

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ABSTRACT

Two improvements that greatly enhance the rate of phosphotriester oligonucleotide synthesis are described: 1) use of hindered primary amines, e.g. t-butyl amine for decyanoethylation of oligonucleotide triester intermediates, and 2) a simplified isolation procedure that eliminates the tedious bicarbonate extraction after each condensing reaction.

Using the improved procedures, oligonucleotide fragments can be synthesized as rapidly as using solid phase chemistry. The final products are purer than those obtained by solid phase chemistry since each intermediate block is purified by chromatography. The technique has been used to synthesize five oligonucleotide fragments (size 15 to 20) for the purpose of performing guided site-specific mutagenesis on a cloned E. coli lipoprotein gene.

INTRODUCTION

The improved phosphotriester method[1] is the most convenient and popular method for the synthesis of deoxyribooligonucleotides in solution. The new phosphite method developed by Caruthers[2] and Ogilvie[3] is suitable only for the solid state synthesis of deoxyribooligonucleotides. Although the phosphite reagent is reactive, the phosphite method has the disadvantage that the reagents may be extremely sensitive to moisture and air.

This report describes two improvements in the phosphotriester method that increase the speed of solution synthesis greatly. The improved procedure can be used to make oligonucleotide 12 to 18 bases long in one day (comparable to the speed of the phosphite solid phase synthesis). In addition, since all the reagents used in phosphotriester synthesis are quite stable, the condensing reaction can be performed in an open atmosphere with consistent results.

The guided site-specific mutagenesis using oligonucleotides as a primer is the most precise and versatile way to introduce specific

mutations in DNA. This technique uses a synthetic oligonucleotide containing a desired mutation (point mutation, deletion or insertion) as a primer to direct the synthesis of DNA on a single-stranded circular DNA template. The oligonucleotide is thereby incorporated into the resulting double-stranded DNA and forms a heteroduplex. Transformation into a host microorganism followed by replication of DNA results in the formation of mutant and parental clones.

The technique has been used successfully first by Smith, et al.[4] to introduce mutations in ϕX174 DNA and later by Wallace, et al.[5] to delete a yeast t-RNA intervening sequence cloned in pBR322. The procedure has been used in generating mutations in a cloned eucaryotic promoter[6], enzyme structural genes,[7,8] the N-terminal signal sequence of E. coli prolipoprotein gene[9], and a human suppressor t-RNA gene[10].

This report focuses on oligonucleotide guided site-specific mutagenesis of the cloned E. coli prolipoprotein gene. The flexible amino acids glycine or proline have been proposed to have an important role in protein secretion[11]. Point and deletion mutations are therefore generated in an area of the lipoprotein signal sequence which codes for two glycine residues in order to study the role of these residues on lipoprotein secretion across the membrane.

RESULTS AND DISCUSSION

Use of Hindered Primary Amines for Decyanoethylation

In modified phosphotriester synthesis[1], the key intermediate is N-protected 5'-dimethoxytrityl-2'-deoxynucleoside 3'-O-chlorophenyl-ß-cyanoethyl phosphates (1). The first step of the standard condensing reaction involves decyanoethylation of (1) with either triethylamine[12] or diisopropylamine[13]. The rate of decyanoethylation of (1d) with various amines was investigated in order to find a better reagent for the reaction. The data summarized in Table 1 indicate that primary amines are generally faster decyanoethylating agents than secondary amines; which, in turn, are faster than tertiary amines.

In order to choose the best decyanoethylating agents two criteria were considered: (i) the amine should be volatile for easy removal by evaporation, and (ii) it should eliminate the cyanoethyl group preferentially over other protecting groups. Since all the amines tested are volatile, the first criterion was met without any further experiment. The selective removal of the cyanoethyl group was then

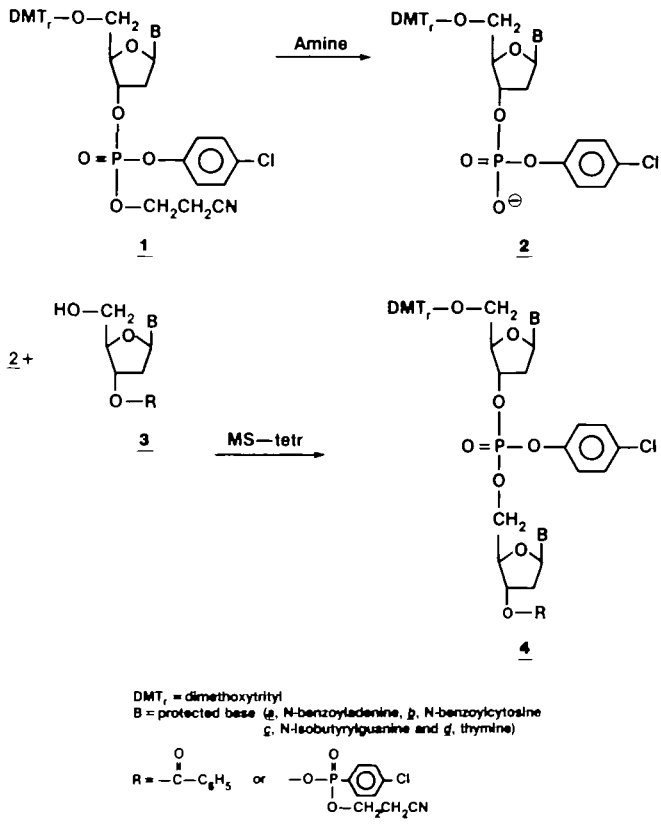

Fig. 1. Decyanoethylation and condensing reactions for improved phosphotriester synthesis

investigated.

Weber and Khorana[14] reported that n-butylamine could remove the N-protecting group on protected nucleosides such as N-protected p-anisolydeoxycytidine (5, Figure 2) with prolonged period of treatment (24 hr). The products are deoxycytidine (6) and the n-butyl derivative of deoxycytidine (7). To investigate whether this undesirable side reaction could occur with the amines used for the decyaneothylation reaction, the reactions of four protected nucleosides with amines were analyzed. The results are summarized in Table 2.

Table 2 shows that protected nucleosides are stable to all the amines tested except that protected deoxycytidine and deoxyadenosine are

TABLE 1
TIME NEEDED FOR COMPLETE DECYANOETHYLATION OF FULLY PROTECTED THYMIDINE

TRIETHYLAMINE	180 MIN.
DIISOPROPYLAMINE	60 MIN.
DIETHYLAMINE	30 MIN.
SEC-BUTYLAMINE	20 MIN.
t-BUTYLAMINE	10 MIN.
n-PROPYLAMINE	2 MIN.

slightly sensitive to n-propylamine. Therefore, n-propylamine is not suitable as a decyanoethylating reagent in triester synthesis, but hindered primary amines such as t-butyl amine or sec-butyl amine are ideal.

Another potential side reaction caused by amines is cleavage of the phosphate protecting chlorophenyl group. To determine if this reaction occurs, we treated a number of protected dinucleotide triester compounds (8) with t-butyl amine-pyridine (1:9, v) solution for 10 min.[15] (Figure 3). The side products from cleavage of chlorophenyl group would be anionic phosphodiester derivatives, which are easily analyzed by hplc or tlc on reversed phase supports. The HPLC results (Figure 4) show that no ionic species are detected under the conditions tested.

New Isolation Procedure

The standard literature[1] procedure for isolating the condensation products employs extensive washing with saturated sodium bicarbonate and H_2O (to decompose the condensing agents) and in vacuo evaporation. A

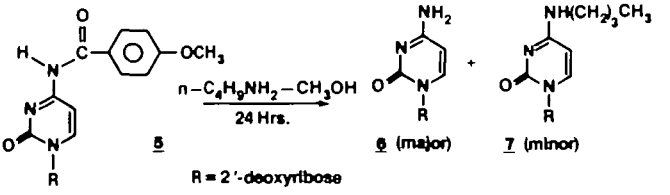

Fig. 2. Reaction of N-anisoyldeoxycytidine with n-butyl amine

TABLE 2
STABILITIES OF N-PROTECTED DEOXYRIBONUCLEOSIDES
WITH VARIOUS AMINES (REACTION TIME 1 HR)

PROTECTED NUCLEOSIDES / AMINES	5'O-DIMETHOXY TRITYLTHYMIDINE	5'O-DIMETHOXY TRITYL-N-BENZOYL DEOXYCYTIDINE	5' O-DIMETHOXY-TRITYL-N-BENZOYL DEOXYADENOSINE	5' O-DIMETHOXY TRITYL-N-ISOBUTYRYL-DEOXYGUANOSINE
TRIETHYLAMINE	N.R.	N.R.	N.R.	N.R.
DIETHYLAMINE	N.R.	N.R.	N.R.	N.R.
DIISOPROPYL AMINE	N.R.	N.R.	N.R.	N.R.
N-PROPYL AMINE	N.R.	TRACE POLAR PRODUCT	TRACE POLAR PRODUCT	N.R.
t-BUTYL AMINE	N.R.	N.R.	N.R.	N.R.
SEC-BUTYL AMINE	N.R.	N.R.	N.R.	N.R.

new isolation procedure was developed by using ether (for trinucleotide or longer) or ether/hexane (1:1,v/v) mixture (for dinucleotide) to precipitate the condensation products. The unreacted condensing reagent mesitylenesulfonoyl tetrazole (Ms-te) and solvent pyridine are soluble in

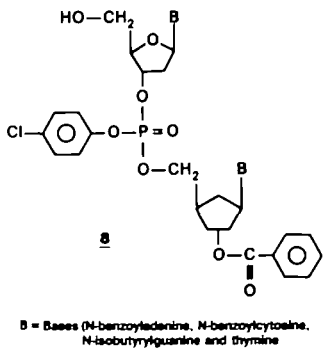

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Fig. 3. Structure of protected deoxyribodinucleotide -3'-benzoate

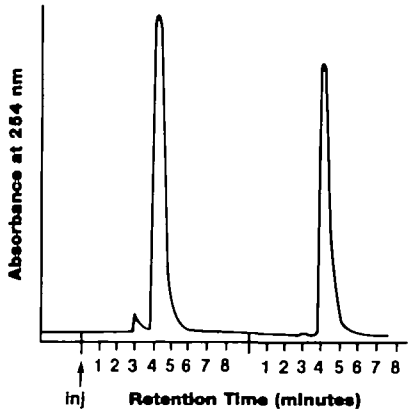

Fig. 4. HPLC chromatogram of protected deoxyribodinucleotide (7b) (left) and the reaction products of 7b with t-butylamine (right)

ether or ether/hexane. By decanting the supernanant, the triester products can be isolated in one step with greater than 90 percent purity.

Using the two modifications, any 12 to 18 base oligonucleotide can be prepared in one day's time starting with protected dinucleotides. Thus, the major work load for oligonucleotide synthesis is now shifted from synthetic chemistry to purification and sequence analysis of oligonucleotide products.

Synthesis of Five Oligonucleotides for the Site-Directed Mutagenesis of the Prolipoprotein Gene

In order to mutagenize two glycine residues in the signal peptide region of the E. coli lipoprotein gene, five deoxyribooligonucleotide fragments were synthesized (Figure 5) using the improved phosphotriester method.

The amino acid sequence of E. coli lipoprotein signal peptide is shown in Figure 5. According to the loop model[11] for protein secretion across a membrane, the glycine residues in position 9 and position 14 of the lipoprotein signal peptide region play an important role in forming a loop that enables the protein to be secreted across the membrane (glycine and proline are present in most β-turns). In order to examine the role of glycine residues, guided site-specific mutagenesis was carried out using the synthetic oligonucleotide shown in Figure 5. Oligonucleotide A_1, a 15-mer, has a mismatch in the codon for gly 9. This mismatch alters gly to val. Oligonucleotide A_2, a 16-mer, has a three base

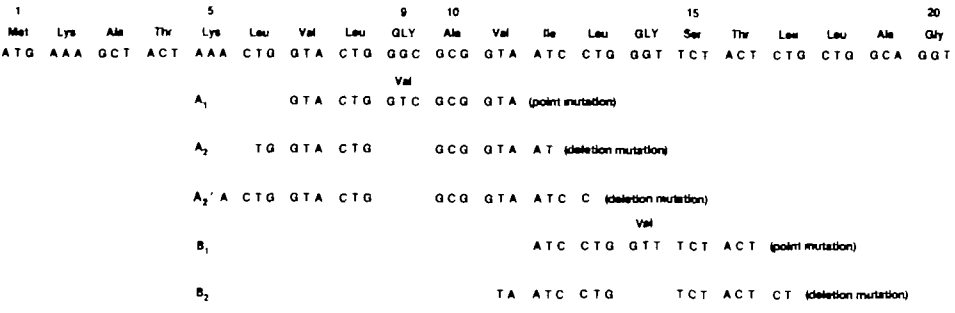

Fig. 5. Signal peptide and corresponding cloned gene sequence of E. coli prolipoprotein. A_1, A_2, A_2, B_1 and B_2 are five oligonucleotide used for guided site-specific mutagenesis study.

deletion at the codon for gly 9. Likewise, oligonucleotide B_1 has a one base mismatch that alters gly 14 to val and B_2 has a three base deletion that will crate a deletion mutant at gly 14. Oligonucleotide A'_2, a 20-mer, also having a three base deletion at gly 9, was synthesized after oligonucleotide A_2 failed to create a deletion mutation.

The general principle of mutagenesis using synthetic oligonucleotides was reported earlier[5]. The mutagenesis was carried out following the method of Wallace, et al.[5] with slight modifications as shown in our previous report[9]. The sequences of five oligonucleotides were confirmed by the mobility shift analysis of Tu, et al.[16]

The desired mutants were detected by colony hybridization using individual ^{32}p-labeled oligonucleotides as probes. The hybridizations were carried out for 16-18 hrs at the following temperature for each probe: A_1, 44°; A_2, 48°; B_1, 38° and B_2, 44° (Figure 6). The yields of positive colonies were 0.2, 0.4 and 0.25 percent of total number of transformants for oligonucleotides A_1, B_1 and B_2, respectively. We were unable to generate mutants with oligonuclotide A_2 for unknown reasons. Therefore, a 20-mer (A'_2) was synthesized and used to create the desirable deletion. Plasmid DNA from positive colonies were isolated and used to re-transform host cells in order to remove wild-type segregants.

Final identification of the mutants was done by DNA sequencing (data not shown). DNA sequences of the mutant DNAs were identical to those predicted from the mutagenesis with the individual oligonucleotides.

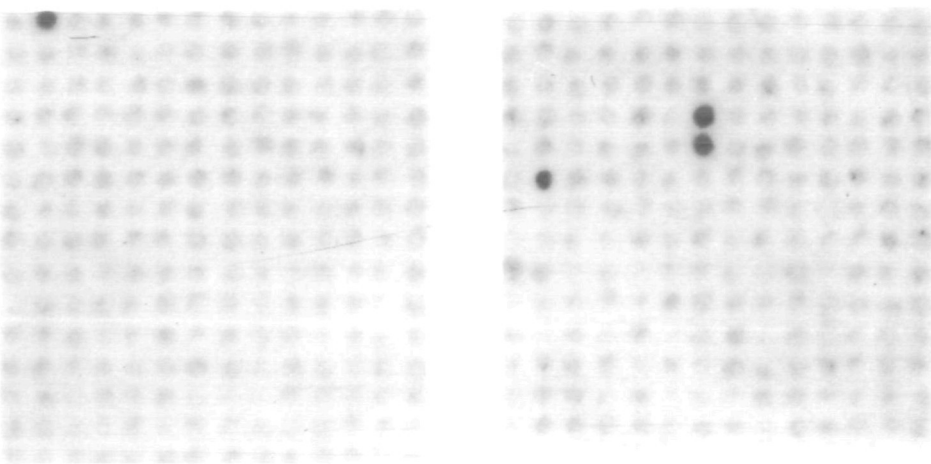

Fig. 6. Colony hybridization of transformants with ^{32}p-labeled oligonucleotides (see MATERIAL AND METHODS for details)

Pulse and Chase Experiments

To study the effect of mutation on the secretion and processing of prolipoprotein, we have first examined the sensitivity of cells transformed with plasmids carrying the mutant lpp genes to a lac inducer, IPTG. It is found that the induction of the A_1 and B_1-lipoproteins is not harmful to the cells, while the induction of B_2-lipoprotein is lethal to the cells. Preliminary pulse-chase experiments are conducted with these mutatnt lipoproteins. At 20 minutes after the addition of IPTG, cells are pulse-labeled for 10 seconds with ^{35}S-methionine and chased for two minutes. The membrane fractions are then treated with anti-lipoprotein serum. The immunoprecipiates are analyzed by SDS-polyacrylamide gele electrophoresis (Figure 7). As shown in Figure 7, the A_1 ($gly_9 \rightarrow val_9$) and B_1 ($gly_{14} \rightarrow val_{14}$) point mutations do not affect the lipoprotein secretion and assembly in the outer membrane; however, the B_2 (gly_{14} deletion) mutation causes significant accumulation of proliportotein in the membrane fraction even after two minutes' chase. It is most likely that the accumulation of the B_2-prolipoprotein causes cell death. More detailed characterizations of these mutants are now in progress. A deletion mutation using oligonucleotide primer A_2 cannot be obtained on gly 9. The longer

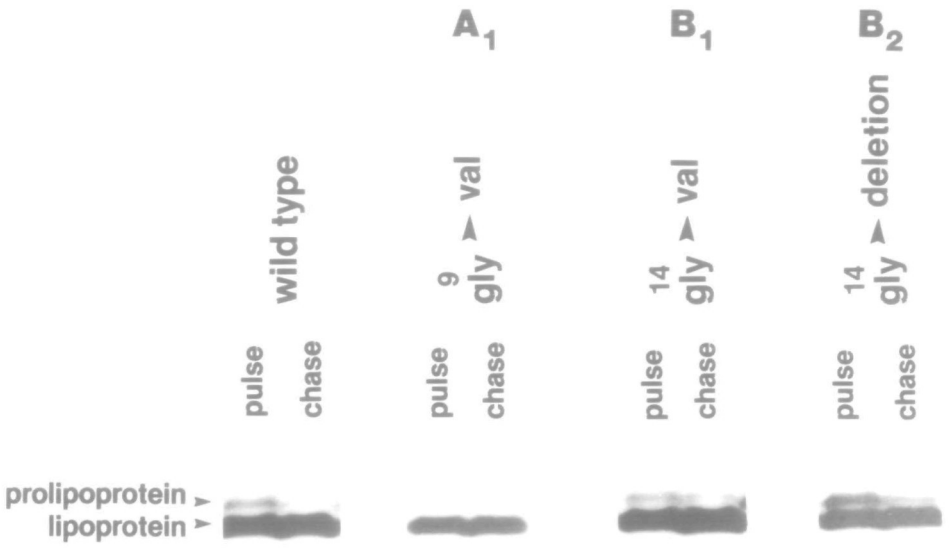

Fig. 7. Autoradiogram of SDS/polyacrylamide gel analysis of immunoprecipitates from outer membrane protein fraction in a pulse-chase experiment (see RESULTS)

oligonuleotide A_2' (20 mer) is therefore used to create the deletion mutation.

MATERIALS AND METHODS

Use of Hindered Primary Amines and Improved Isolation Procedure

Fully protected mononucleotides were prepared following a published procedure[1]. Fully protected mononucleotide 1 (150 mg, 1a) was dissolved in 1 ml of pyridine and approximately 0.1 ml of t-butyl amine was added. The decyanoethylation reaction was complete in 7 min. as shown by silica gel tlc with CH_3OH - CH_2Cl_2 (1:10, v/v). At that time, 30 ml of an ether/hexane (1:1) mixture was added and the precipitate formed immediately. The precipitate was recovered by centrifugation in a 40 ml centriguge tube and the supernatant was decarded. The precipitate was then dissolved in CH_2Cl_2 and evaporated to dryness in preparation for the condensing reaction. In the condensing reaction (Figure 1) the compound 2 was combined with the compound 3 (100 mg) in 5 ml of dichloromethane. The combined 2 and 3 solution was then evaporated to dryness in vacuo. The dried compounds

were then redissolved in 1.5 ml of anhydrous pyridine. The condensing reagent mesitylenesulfonyl tetrazole (Ms-te) was then added and the reaction was compelte in 20 min. The reaction product was precipitated with 30 ml of ether/hexane (1:1, v/v). The precipitate was recovered by centrifugation and chromatographed on silica gel thin layer plates (CH_3OH/CH_2Cl_2, 1:10, v/v). The yield of 4 was 200 mg (85 percent).

Longer oligonucleotide triesters were prepared by a similar procedure except that anhydrous ether was used instead of the ether/hexane mixture for precipitation. The synthetic oligonucleotide triester was deblocked by the standard procedure[1] or oximate procedure[18] and purified on Polyethyleneimine impregnated cellulose tlc (PEI)[1]. The PEI purified oligonucleotides were then eluted[1] and purified further by high performance liquid chromatography on a μ Bondpack C18 column (Waters)[17].

5'^{32}p-Phosphorylation of Oligonucleotides

Oligonucleotides were labeled following the well-established procedure[1] using [γ-^{32}p]ATP (>2000 Ci/mmole) from Amersham and polynucleotide kinase from P-L Biochemicals. Labeled oligonucleotides prepared in low specific activity (200 Ci/mmole) were used as primers for site-directed mutagenesis and high specific activity (2000 Ci/mmole) oligonucleotides were used as hybridization probes. The labeled oligonucleotides were purified by Sephadex G-50 (superfine) chromotography using 20 mM triethylammonium bicarbonate (TEAB) buffer (pH 8.0) and lyophilized to a dry powder.

Bacterial Strains and Plasmids

The bacterial strains used were described previously[9].

Plasmid pKEN125 (3.9 kb) was used for guided site-specific mutagenesis. This plasmid was derived from pBR322 and carries an inducible *lpp* gene. The plasmid construction was described earlier[9]. JA221 *lpp*$^-$/F'*lacI*^{q} was used as a recipient and, in the absence of *lac* inducer, the production of the lipoprotein was negligible. The addition of isopropyl-β-D-thiogalactoside (IPTG;Sigma) induces lipoprotein production to about 4 times that of the lpp+ wild-type cells.

Directed Site-Specific Mutagenesis

Directed site-specific mutagenesis using oligonucleotides was performed following the published procedure[5,9]. The nicking of plasmid pKEN125 DNA was monitored by 0.7 percent agarose gel electrophoresis. Electrophoresis showed that greater than 90 percent covalently closed circular DNA became open circular form by the nicking procedure.

Five μg of nicked pKEN125 was incubated in 20 μl Exo III buffer (66 mM Tris-HCl pH8, 0.66 mM $MgCl_2$, 1mM β-mercaptoethanol) with 40 units of EXO III (New England Biolabs) for 90 min. at 37°C. The salt concentration in the reaction mixture was then adjusted to 6mM Tris-HCl, 6 mM $MgCl_2$, 50 mM NaCl, and 6 mM β-mercaptoethanol. Then 2.8 μl Hinf I enzyme (New England Biolabs) was added and after 30 min. incubation, EDTA was added to quench the reaction (final concentration 10 mM). The reaction mixture was extracted twice with phenol, twice with ether, and single-stranded DNA was recovered by ethanol precipiation after addition of sodium acetate (final concentration 0.3M) and $MgCl_2$ (0.025M). The DNA was then redissolved in 20 μl buffer (10 mM Tris-HCl, pH 8.0) and 8 units of bacterial alkaline phosphatase (Worthington) was added. The mixture was incubated at 37°C for 30 min. The phenol and ether extraction steps were then repeated before precipiation of the DNA with ethanol.

The single-stranded DNA (5 μg) was resuspended in 60 μl polymerase-ligase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM β-mercaptoethanol), 90 pmoles of 5'-phosphorylated oligonucleotide primers were added, the reaction mixture was heated to 90°C for 3 min. and then cooled to 4°C for annealing. After 2 hrs at 4°C, the reaction mixture was adjusted to 0.5 mM in each of the deoxynucleotide triphosphates. ATP (1.25 mM) and DNA polymerase (large fragment, Boehringer-Mannheim) 90 units/ml and T_4 DNA ligase (BRL, 100 weiss units/ml) was added and the mixture incubated at 12°C overnight.

Transformation and Screening for Mutants

The DNA from the oligonucleotide primed reaction was used to transform JA221 $lpp^-/lac\ I^q$ by a published procedure[9]. The transformed bacteria were plated on 100 mm L-broth agar plates containing 50 μg/ml ampicillin. Ordered collections of colonies were picked onto 100 mm L-broth agar plates containing 50 μg/ml ampicillin (~60 colonies per plates). After overnight growth, colonies were transferred to square Whatman 540 filter paper, grown overnight on L-broth Agar plates containing 50 μg/ml ampicillin. The filter was then removed and colonies were lysed and hybridized with ^{32}p-labeled oligonucleotides (8 x 10^6 cpm per filter). The hybridizations were performed in a buffer that contained 6x NET (NET=0.15M NaCl, 0.015M Tris-HCl (pH 7.5), 0.001 M EDTA), 5x Denhardts, 10 percent Dextran sulfate (Sigma) and 0.5 percent SDS. The hybridizations were carried out at 44°C (A_1), 48° (A_2),

38°C (B_1), 44°C (B_2) for 16 hrs. Filters were then washed in 6x SSC (SSC = 0.15M NaCl, 0.015M Na Citrate) at ambient temperature and exposed to Kodak XR-5 Xray film. The results are shown in Figure 6.

Pulse-Chase Labeling and SDS-Polyacrylamide Gel Electrophoresis SDS

Pulse-chase labeling experiments were performed on mutated E. coli cells. SDS polyacrylamide gel electrophoresis was performed on immunoprecipitates of membrane protein fractions by anti-lipoprotein anti-serum as described previously[9] and the results are shown in Figure 7.

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